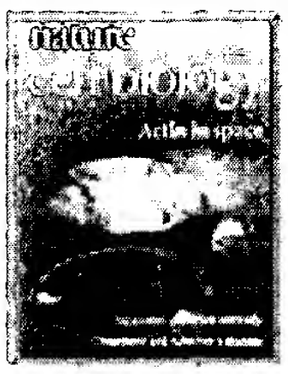

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Borg proteins control septin organization and are negatively regulated by Cdc42

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The Cdc42 GTPase binds to numerous effector proteins that control cell polarity, cytoskeletal remodelling and vesicle transport. In many cases the signalling pathways downstream of these effectors are not known. Here we show that the Cdc42 effectors Borg1 to Borg3 bind to septin GTPases. Endogenous septin Cdc10 and Borg3 proteins can be immunoprecipitated together by an anti-Borg3 antibody. The ectopic expression of Borgs disrupts normal septin organization. Cdc42 negatively regulates this effect and inhibits the binding of Borg3 to septins. Borgs are therefore the first known regulators of mammalian septin organization and provide an unexpected link between the septin and Cdc42 GTPases.

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The Rho-family GTPase Cdc42 regulates many essential biological processes including cell polarity, cytokinesis, vesicular transport and cytoskeletal remodelling[1-5]. Regulation is mediated by specific effector proteins, many of which contain a Cdc42/Rac interaction binding (CRIB) motif that is recognized specifically by the GTPase in the GTP-bound state[6]. We have previously described a family of five putative effectors for Cdc42, which we called Borgs[7]. The Borg proteins share several regions of similarity: a CRIB motif through which they bind the GTPase, and three Borg-specific domains, BD1, BD2 and BD3. Borg3 is unique in that it does not efficiently bind the related GTPase, TC10, and does not contain a BD2 domain. When expressed ectopically in mammalian cells, Borg3 can cause cell-shape remodelling and delays in cell spreading on fibronectin[7, 8].

The signalling pathways through which Borgs operate are not known. We consequently sought binding partners for the Borgs that might indicate their cellular functions. We found that Borgs interact with a complex of septin GTPases, specifically via their BD3 domains. Remarkably, therefore, the Borgs link two distinct families of GTPases. Septin GTPases are thought to regulate cytokinesis, vesicular transport and cell polarity[9-12] (reviewed in refs 13,14). Septins have also recently been shown to be involved in oncogenesis (reviewed in ref. 15). The *MSF* septin gene is deleted in breast and ovarian cancers and has been proposed to be an anti-oncogene[16]. The *CDCrel-1* septin gene is deleted in some velo-facio DiGeorge syndrome patients[17]. Products of both septin genes are also found fused in a carboxy-terminal position to the mixed-lineage leukaemia (MLL) protein in acute leukaemia[18, 19].

Septins are able to form multimeric complexes[20-22] and Borgs can markedly alter their organization within the cell. Cdc42 inhibits the association of Borg3 with septins. Thus, Borgs are the first known regulators of septin organization in mammalian cells and also provide a first example of a CRIB domain effector that is inhibited rather than activated by Cdc42-GTP.

Results

Borg3 binds to septin GTPases. To seek Borg functions we used Borg3 fused to glutathione *S*-transferase (GST–Borg3) bound to glutathione–Sepharose beads as an affinity matrix for proteins from NIH 3T3 fibroblasts labelled with [^{35}S]methionine. Four major bands were specifically recovered (Fig. 1a). When repeated on a larger scale, using thrombin to cleave the GST–Borg3, these four proteins were detected by staining with Amido Black (Fig. 1b). Recovered proteins were digested by CNBr or skatol and the mixed peptides were directly sequenced without purification, then sorted and matched against databases by using the FASTF algorithm[23]. One band was identified as the heat shock protein Hsp70 (Fig. 1b, P1) and was not studied further. The other major proteins were all identified as septins: P2 as Septin6, P3 as Cdc10 and P4 as Nedd5 (Fig. 1b, c). Two fainter bands, P5 and P6, were identified respectively as MSF/E-Septin[18, 24] and as a heavier form of Cdc10. (Peptides from the lower, M_r 27K, band in Fig. 1b corresponded to GST.)

In *Saccharomyces cerevisiae*, two subunits each of septins Cdc3, Cdc10, Cdc11 and Cdc12 form hetero-octamers of M_r ~370K in size, as well as filaments[20]. In *Drosophila*, Pnut, Sep1 and Sep2 septins combine to form heterohexamers (M_r 340K) and filaments[21, 22]. Cdc10, Nedd5, Septin6 and H5 septins purify together from mammalian brain extracts with the Sec6/Sec8 exocyst protein complex[25], and anti-Nedd5 antibody labels short filamentous structures in brain and HeLa cells (refs 10,26 and unpublished data). Our affinity purification of septins at a stoichiometric ratio of 1:1.2:1.6 suggests that Nedd5, Septin6 and Cdc10 might exist as a hetero-oligomer. This hypothesis was supported by size-exclusion chromatography, which showed that the purified septins eluted as a broad peak of high relative molecular mass (data not shown). To test whether one, several or all of the septins recovered in the pull-down assay could bind directly to Borg3, we tried to express septins in *Escherichia coli*. However, only Nedd5 could be produced in this way (Fig. 1d). Septin6 and Cdc10 were insoluble unless expressed together from a bicistronic vector. By using an additional vector, we could produce all three septins simultaneously (Fig. 1d). GST–Borg3 did not associate efficiently with Nedd5 from bacterial extracts but did bind Cdc10 + Septin6 (Fig. 1e). Borg3 therefore binds directly either to Septin6 or Cdc10, or to Septin6/Cdc10 heterodimers, and no bridging proteins are required for the interaction. This property is not unique to Borg3 because both GST–Borg1 and GST–Borg2 were also able to bind Cdc10/Septin6 (Fig. 1f).

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The BD3 domain is responsible for septin binding. To map the region of the Borgs required for septin association we used GST–Borg3 constructs spanning different regions of the protein, and ^{35}S-labelled NIH 3T3 cell lysate (Fig. 2a). A fragment encompassing the BD3 domain, GST–Borg3(83–110), was necessary and sufficient to precipitate septins (Fig. 2c). To confirm specificity, we generated full-length GST–Borg3 proteins containing mutations within the BD3 domain (Fig. 2b), namely GST–Borg3(G98A, P99A, S100A) and GST–Borg3(L102A, V105A, L106A). Neither of these proteins could bind septins (Fig. 2c). We next produced a rabbit polyclonal antibody directed against a peptide corresponding to the C-terminus of Borg3 (amino acids 137–150). This antibody was specific for the Borg3 isoform because it detected Myc–Borg3 or triple-haemagglutinin-1 (HA_3)-tagged Borg3 expressed in COS-7 cells, but not Borg1 (Fig. 3a). Immunoblots with the anti-Borg3 antibody detected the endogenous protein in NIH 3T3 and Madin–Darby canine kidney (MDCK) cells but not in Chinese hamster ovary, COS-7 or HeLa cells (Fig. 3b). To test the ability of Borg3 to associate with endogenous septins, we expressed untagged Borg3 in COS-7 cells and immunoprecipitated the protein with the anti-Borg3 antibody. Both endogenous Nedd5 and Cdc10 were recovered in the precipitate. A similar result was obtained with wild-type HA_3–Borg3, but no septins were immunoprecipitated together when cell lysates containing the mutants HA_3–Borg3(GPS) or HA_3–Borg3(LVL), which are unable to bind septins, were used (Fig. 3c). Taken together, these data demonstrate that Borg3 binds to septins via its BD3 domain. Importantly, the anti-Borg3 antibody was able to precipitate endogenous Borg3 with endogenous Cdc10 from a rat brain extract (Fig. 3d), confirming that this interaction is of physiological relevance.

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Borgs can disrupt normal septin organization in the cell. If Cdc10, Nedd5 and Septin6 septins form hetero-oligomers and filaments, they should show identical intracellular distributions. To examine this question, MDCK cells were labelled with anti-septin antibodies and examined by epifluorescence microscopy. Nedd5 localized with both Cdc10 and Septin6. Septin labelling was revealed as dots or short filaments throughout the cytoplasm, with a different pattern in fibroblast-like cells (Fig. 4b) from those forming an epithelial monolayer (Fig. 4a). We confirmed a partial localization of septins with actin stress fibres[10], and also observed partial localization with the tubulin network (data not shown). We considered it unlikely that localization of endogenous Borg3 with septins would be easily detectable, because their interaction is probably regulatory rather than constitutive, and the anti-Borg3 antibody is not sensitive enough to be used for immunofluorescence. We therefore could not address this question.

As we reported[7], Myc–Borg3 is distributed throughout the cell (Fig. 5d). Interestingly, however, the expression of Myc–Borg3 (or HA_3–Borg3) often induced the formation of long or thick septin fibres, an effect that was particularly noticeable when the transfected cells were compared with neighbouring, untransfected ones (Fig. 5d–f). This effect on septin organization might be due to bundling or to extension by polymerization of the septin filaments.

The isolated BD3 domain, expressed as a fusion protein with a triple green fluorescent protein tag, GFP_3–BD3, provoked a total aggregation of septins, usually at a single perinuclear spot, where GFP_3–BD3 was also concentrated (Fig. 5g–i). The mutant GFP_3–BD3(LVL), which is defective in septin binding, did not induce this phenotype (Fig. 5j–l): the transfected cells were undistinguishable from those expressing GFP_3 alone (Fig. 5a–c). Similar immunofluorescence data were obtained from other cell types, including NIH 3T3 or baby hamster kidney (BHK) (data not shown). In all cases, septin reorganization had no observable effect on the actin or tubulin networks or on adherens or tight junctions, indicating that these structures are independent of the septin status in the cell (data not shown).

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Overall, our results demonstrate that the association of Borg3 with septins can alter their organization state. We infer that the association is regulated, because, in the presence of the full-length protein, aggregates of septins were rarely observed (compare Fig. 5f with Fig. 5i). Surprisingly, however, the expression of GFP–Borg3 induced a phenotype similar to that induced by GFP_3–BD3, and different from that of Myc–Borg3 (Fig. 5m–o). A similar result was obtained with GFP–Borg1, although total aggregation was less commonly detected (Fig. 5p–q). These data suggest that a large epitope tag at the amino terminus of Borgs might modify the structure of the protein so as to expose the BD3 domain, thereby enhancing its affinity for septin complexes and promoting uncontrolled septin-polymerization/bundling. This mechanism might also account for the efficiency with which GST–Borg3 beads accumulate septins from cell lysates (Fig. 1b). We speculate that, like N-WASP[27], Borg3 might function as a conformational switch, in which exposure of the BD3 domain is regulated by association with another factor. In this model, the large N-terminal tags on GST–Borg3 or GFP–Borg3 would force a constitutive exposure of the BD3 domain. However, further work will be necessary to test this idea. We have so far been unable to induce septin aggregation *in vitro* upon the addition of a GST–BD3 domain fusion protein, to purified septin filaments from rat brain or to recombinant septins. *In vitro* reconstitution might require factors that alter the GTP/GDP binding state, or some other state, of the septin monomers.

Cdc42-GTP inhibits septin binding to Borg3. We have shown that Borgs bind to two types of GTPase: septins and Cdc42. To understand whether and how Cdc42 regulates the Borg–septin interaction, we performed immunoprecipitations from cells that expressed various forms of Cdc42. Surprisingly, dominant-negative Cdc42(T17N) had no effect on the precipitation of the Cdc10 septin with HA_3–Borg3, whereas an activated mutant, Q61L, decreased the binding of Borg3 to Cdc10 in a dose-dependent manner (Fig. 6a). Wild-type Cdc42 had an intermediate effect. Activated Cdc42 had no effect when co-expressed with a mutant of the CRIB domain of Borg3 that cannot bind Cdc42 (ref. 7) (Borg3($I_{23}A$, $S_{24}A$); Fig. 6a, right panel). Inhibition of septin binding to Borg3 could also be observed *in vitro* by using GST–Borg3 and a bacterial extract containing Septin6 and His_6–S-Cdc10. The addition of His_6–Cdc42(Q61L)-β-γ-imidoguanosine 5'-phosphate (GMP-PNP) decreased the association of His_6–S-Cdc10 with GST–Borg3 (Fig. 6b). Moreover, although GFP–Borg3 behaves as though it were constitutively active (Fig. 5), it is still sensitive to the inhibitory effect of Cdc42(Q61L) (Fig. 6c), indicating that Cdc42-GTP can bind to the CRIB motif of Borg3 in the 'open' state and displace septins attached to the BD3 domain. This negative regulatory mechanism seems to operate in intact cells, because the expression of activated Cdc42 in MDCK cells induced a loss of septin filaments (Fig 7a, b). Gain-of-function Cdc42

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seems to cause a redistribution of the septins into a vesicular pattern localized mainly in the perinuclear region. This effect is maintained when the Cdc42 is expressed with wild-type Borg3 (Fig. 7c, d; 82% of cells show no, or fewer, septin filaments than surrounding untransfected cells), suggesting that the Cdc42 can suppress Borg3-mediated septin reorganization. However, when expressed with a CRIB mutant of Borg3, which cannot bind Cdc42, septin filament formation does occur (Fig. 7e, f, 77% of cells show as many or more septin filaments than neighbouring untransfected cells). This result suggests that Cdc42-GTP inhibits septin filament reorganization by binding to and inhibiting Borg3.

Discussion

Septins are essential components of budding yeast and are necessary for the completion of cytokinesis in mammalian cells. They might also function in exocytosis and have been implicated in several types of cancer. They form a novel class of GTPases. Despite their importance, however, the molecular functions of septins in metazoans, at least, have remained obscure, and no mammalian regulators of septin GTPase activity or organization have been identified. The Borgs represent the first such regulators of septin organization to be discovered in metazoans, and link septin GTPases to a signal transduction pathway mediated by the GTPase Cdc42. This pathway has no obvious counterpart in budding yeast, the genome of which does not contain Borg genes, but a functionally similar connection with septins might exist because a Cdc42p effector mutant disrupts the septin ring structure at the yeast bud emergence site[2]. In this case the connection might occur through Cla4p, a downstream effector kinase for Cdc42p in yeast, which regulates the Gin4p kinase, which in turn promotes septin ring formation[22, 28]. Borg genes also seem to be absent from fruitflies, but interestingly the *Drosophila* genome contains an Ack-like tyrosine kinase gene that contains CRIB, BD1 and BD3 domains near its C-terminus. Ack is a downstream effector of Cdc42 (ref. 29), so Ack and Borg functions in flies might be partly fused into one protein.

How is the Borg–septin interaction controlled? Other effectors of Cdc42 that contain a CRIB motif—WASP, N-WASP and PAK—have been shown to function as conformational switches[30-33]. In isolation these proteins are in an inactive, closed state. Binding of Cdc42-GTP can trigger deployment to an active, open state. For N-WASP this trigger requires a second signal input, provided by a phospholipid, phosphatidylinositol 4,5-bisphosphate[34, 35]. Here we describe an inverse mechanism, in which the binding of Cdc42-GTP to the CRIB motif of Borg3 disrupts interaction with its target. However, our studies suggest that Borg3 might nevertheless exist in a closed or open state, because the expression of Myc-tagged Borg3 has only a minor effect on septin organization but the expression of either the isolated BD3 domain or GFP–Borg3, which possesses a large N-terminal tag, causes a marked aggregation of septins. It is unlikely

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that the GFP tag causes misfolding of the Borg3 because it is still capable of binding Cdc42. We speculate, as a working model, that Borgs3 is normally inactive but that the binding of a regulatory factor—or, artificially, the attachment of a large N-terminal tag—forces the protein into the open state that can interact with the septins and trigger their re-organization. Association with Cdc42-GTP then inhibits this interaction. Further studies will be necessary to address how Borgs modify the organization of septin complexes and what additional factors control Borg function. The ability to express septin oligomers in bacteria will facilitate this work. Moreover, the identification of Borgs as the only known mammalian regulatory factors for septin organization should aid in the explanation of septin functions in the mammalian cell.

Methods

Pull-down assays. GST fusion proteins were immobilized on glutathione–Sepharose beads and incubated with cell lysates. NIH 3T3 cells were labelled with [^{35}S]methionine (Tran^{35}Slabel; ICN, 0.4 mCi/100-mm plate). Large-scale purification was performed with ~3 × 10^8 NIH 3T3 cells and 2.5 mg of GST–Borg3 proteins. Cells were lysed in 150 mM NaCl, 50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.5% Triton X-100 and protein inhibitor cocktail (Sigma). The cell extracts were centrifuged and the supernatants were precleared with glutathione–Sepharose beads and incubated with the GST–Borg3 for 20 min. Beads were washed with the same buffer, then with buffer without detergent. Proteins were eluted with 20 mM glutathione. GST–Borg3 was digested with thrombin[36]. Proteins were then separated, digested and identified as described previously[23]. Borg3 N- and C-terminal deletion mutants were generated by polymerase chain reaction. Borg3(GPS) and (LVL) mutants were made with the Quick-Change mutagenesis kit (Stratagene). All constructions were checked by sequencing before use. Methods for the cloning, expression and purification of recombinant septins are available from the authors on request.

***In vitro* binding assay.** GST or GST–Borg3 (1 μg), bound to glutathione–Sepharose beads, was incubated with or without purified recombinant Septin6/His_6–S-Cdc10 dimer (4 μg) and His_6–Cdc42(Q61L) previously loaded with the slowly hydrolysable GTP analogue GMP-PNP (4 μg) in reaction buffer (25 mM HEPES pH 7.4, 100 mM NaCl, 0.5% Triton X100, 1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol) for 1 h at 4 °C. Precipitates were then washed four times with the same buffer and processed for immunoblotting as described below.

Immunoprecipitations. All polyclonal antibodies were affinity-purified. COS-7 cells were transfected and lysed, and the extracts were subjected to immunoprecipitation as described previously[1,7]. Immunoprecipitates were washed three times in lysis buffer (150 mM NaCl, 25 mM HEPES pH 7.4, 0.5 mM EDTA, 1 mM

$MgCl_2$, 1 mM phenyl methylsulphonyl fluoride, 0.5 mM DTT, 0.5% Triton X-100) then once in the same buffer without Triton. Brain extract was prepared from one rat brain homogenized in the same lysis buffer but containing 10 mM 2-mercaptoethanol. After centrifugation (20 min at 18,000*g*) the pellet was resuspended in lysis buffer containing 1% Triton X-100 and centrifuged again. The supernatant was then diluted 1:1 with lysis buffer without Triton X-100 and mixed with the first supernatant. Immunoprecipitates were washed as described above. In all experiments, proteins were separated by SDS–PAGE and detected with specific antibodies either directly coupled to horseradish peroxidase (HRP) (anti-HA, anti-Myc and anti-S-peptide mouse monoclonals) or revealed by HRP-conjugated anti-rabbit (for anti-Nedd5 (ref. 26), anti-GST, anti-GFP, anti-Borg3 and anti-His) or anti-guinea-pig (for anti-Cdc10 and anti-Septin6 (ref. 26)) secondary antibodies. Proteins were then revealed by chemiluminescence (Kirkegaard & Perry).

Immunofluorescence. MDCK cells were grown in LabTek chambers (Nunc) and transfected (for 7–9 h) with Effectene (Qiagen) as described previously[1]. Cells were fixed for 24–30 h after the beginning of transfection with 4% paraformaldehyde in PBS, quenched with NH_4Cl and permeabilized with 0.2% saponin in PBS as described previously[7]. All washes and incubations were done in TBS–0.05% Tween 20. Cells were then blocked for more than 20 min with 10% FCS, incubated with the first antibody for 1 h, washed, and incubated with the secondary antibody coupled to Oregon Green or Texas Red for 45 min. Cells were mounted and imaged as described previously[1]. To quantify Cdc10 organization, three groups of at least 100 cells each, from two independent experiments, were analysed for each condition. In Fig. 6, Nedd5 organization was quantified for 100 cells; a parallel immunofluorescence study was performed to check that close to 100% of transfected cells were expressing both Borg3 and Cdc42 proteins. For all data sets, the phenotypes of transfected cells were compared with those of neighbouring, untransfected cells.

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This PDF replaces the previous online version published on August 31 2001. Label on Figure 2c has changed from (1-150)GPS-AAA to (1-150)LVL-AAA.

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